Rapid determination of the metabolic stability of pharmaceutical drug candidates by triple quadrupole mass spectrometry

Method development and determination of metabolic stability using an Agilent 1200 Series RRLC system and an Agilent 6410 triple quadrupole LC/MS

Application Note

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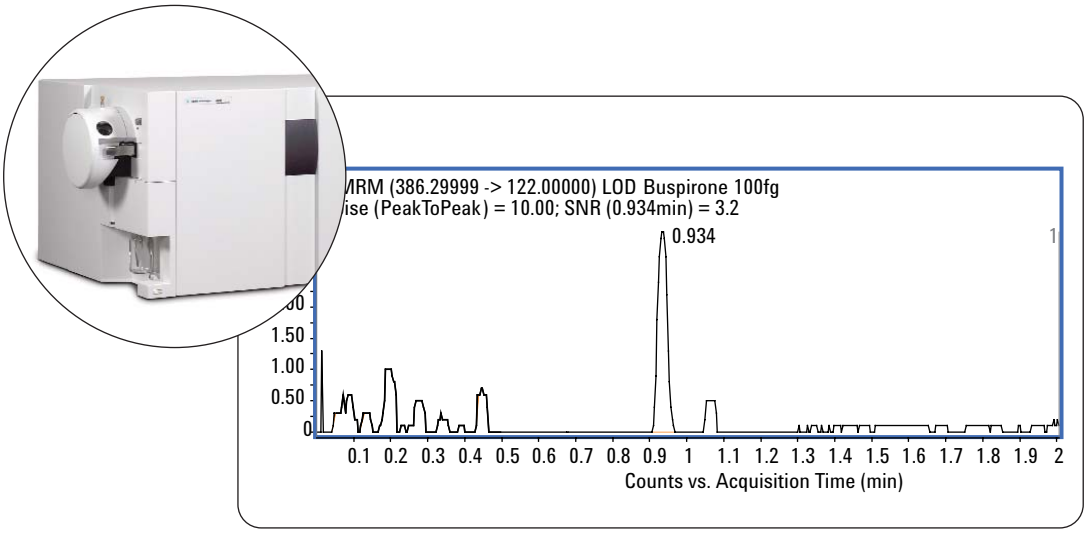

Abstract

This Application Note describes:

- The rapid development of a MRM method for triple quadrupole mass spectrometry.
- The rapid development of a liquid chromatography method for high throughput conditions with a 1.8 µm RRHT column.
- The use of the developed LC/MS MRM method for rapid determination of a metabolic stability profile of a pharmaceutical drug.
- The relative quantification of the acquired data to generate the metabolic stability profile.

Agilent Equipment:

1200 Series RRLC
6410 triple quadrupole LC/MS
ZORBAX RRHT column
MassHunter workstation software

Application Area:

Early ADME in drug discovery and development

Agilent Technologies

Introduction

In modern pharmaceutical drug development it is of crucial importance to know the adsorption, distribution, metabolism and excretion (ADME) behavior of a new drug substance. Therefore, a recent trend in the drug discovery and development process is to shift the starting point of drug metabolism and pharmacokinetic (DMPK) studies to a point as early as possible in the development chain to address potential issues in parallel with the optimization of the drug's lead structure[1].

To address this need, many in-vitro frontline assays are available to identify potential problems in the development of a new drug substance. A typical experiment is the determination of the drug's metabolic stability. In this type of in-vitro metabolism experiment the relative concentration of a parent drug, which decreases over time, is monitored[2]. To examine the large amount of samples it is necessary to develop a fast, high resolution LC method in combination with a selective MRM triple quadrupole MS method, which is capable to fulfill high throughput requirements. In this Application Note we demonstrate the rapid development of a fast, high resolution LC method together with a selective MRM triple quadrupole MS method for the determination of the metabolic stability profile of pharmaceutical drugs.

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution LC (RRLC) system
- Agilent ZORBAX SB C-18, 2.1 x 50 mm, 1.8 µm particle size
- Agilent 6410 triple quadrupole LC/MS system
- Agilent MassHunter Workstation for data acquisition and qualitative data analysis

MRM LC/MS method

- Agilent 1200 Series SL pump
  Solvent A: water + 0.1 % formic acid (FA)
  Solvent B: ACN + 0.1 % formic acid (FA)
  Flow: 1 mL/min
  Gradient: 10-55 %B in 2 min
  Stop time: 2 min
  Post time: 2 min.
- Agilent 1200 Series SL autosampler
  Injection volume: 1 µL
  Sample temperature: 4 °C
  Needle wash: 5 s in wash port with methanol
  Sample flush out factor: 20
  Automated delay volume reduction after injection and flushing by switching the injector loop to bypass
- Agilent 1200 Series SL column compartment
  Column temperature: 50 °C.
- Agilent 1200 Series variable wavelength detector (VWD)
  Detection wavelength: 200 nm
  Peak width: 0.25 s
  Flow cell volume: 4 µL
- Agilent 6410 triple quadrupole LC/MS system
  Source: ESI, positive
  Temperature: 300 °C
  Gas flow: 12 L/min
  Nebulizer pressure: 60 psi
  Capillary voltage: 4000 V
  MRM settings for each time segment: parent ion mass –> quantifier ion mass (386.5 –> 122.0)
  Dwell time: 35 ms
  Fragmentor voltage: 180 V
  Collision energy: 35 V

Flow injection method for optimization of fragmentor voltage

- Agilent 1200 Series SL pump
  Flow: 0.5 mL/min
  Solvent: 65 % A, 35 % B
  Stop time: 5 min
- Agilent 1200 Series SL autosampler
  Injection volume: 1 µL
  Sample temperature: 4 °C
  Injector program:
  Remote Start pulse,
  Repeat 9 times,
  Valve bypass,
  Draw sample,
  Valve mainpass,
  Wait 0.25 min,
  End repeat.
- Agilent 6410 triple quadrupole LC/MS system
  Source: ESI, positive
  Temperature: 300 °C
  Gas flow: 12 L/min
  Nebulizer pressure: 60 psi
  Capillary voltage: 4000 V
  Time segments: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 min
  MS2 Scan mode: 100-400 m/z
  Scan time: 200 ms
  Fragmentor voltages according to the time segments: 60, 80, 100, 120, 140, 160, 180, 200 and 220 V

Flow injection method for optimization of collision energy

- Agilent 1200 Series SL pump
  Flow: 0.5 mL/min
  Solvent: 65 % A, 35 % B
  Stop time: 6 min
- Agilent 1200 Series SL autosampler
  Injection volume: 1 µL
  Sample temperature: 4 °C

Injector program:
Remote Start pulse
Repeat 11 times
Valve bypass
Draw sample
Valve mainpass
Wait 0.25 min
End repeat

- Agilent 6410 triple quadrupole LC/MS
  Source: ESI, positive
  Temperature: 300 °C
  Gas flow: 12 L/min
  Nebulizer pressure: 60 psi
  Capillary voltage: 4000 V
  Time segments: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 min
  Product Ion
  Scan mode: 100-400 m/z
  Scan time 200 ms
  Optimized fragmentor voltage: 180 V
  Collision energy according to the time segments: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 V

Sample preparation

- Stock solutions:
  20 mg/mL S9 preparation
  0.1 mg/mL buspirone in water
  1.6 mg NADP in 1.6 mL 0.1 M phosphate buffer pH 7.4
  50 mM isocitrate/$MgCl_2$ (203 mg $MgCl_2$ x $6H_2O$ + 258.1 mg isocitrate in 20 mL H_2O) Isocitrate dehydrogenase 0.33 unit/µL
- NADPH regeneration system:
  1.6 mL NADP solution + 1.6 mL isocitrate solution + 100 µL IDH solution
- Incubation mixture:
  3.85 µL substrate + 200 µL NADPH regeneration system + 746.15 µL phosphate buffer + 50 µL S9

Incubation was carried out at 37 °C for 30 minutes and a 100 µL aliquot was taken every 5 minutes. The reaction was stopped by adding 6 µL perchloric acid and 100 µL acetonitrile followed by centrifugation for 15 min at 14,000 g. The supernatant was evaporated to dryness using a SpeedVac concentrator and reconstituted with water containing 0.1 % formic acid for LC/MS analysis as described below. Incubations stopped at 0 min were used as controls.

Results and discussion

For the relative quantitative determination of a drug compound within a complex matrix such as in a metabolism experiment of a drug, it is necessary to have a method with very high selectivity for the substance. Therefore, for such experiments triple quadrupole mass spectrometers are used in MRM mode where the precursor ion is isolated in the first quadrupole and after fragmentation in the collision cell a specific fragment is isolated in the last quadrupole. This transition from precursor to fragment ion gives enough specificity and sensitivity for quantification. To get optimum performance in sensitivity it is necessary to adjust the fragmentor voltage of the instrument for highest signal intensity of the precursor ion and subsequent optimization of the collision energy gives the highest intensity for the main fragment.

To achieve this optimum performance a solution of the compounds at a concentration of 1 µg/mL was prepared. For the optimization of the fragmentor voltage and collision energy the described flow injection methods with an injection of 1 ng on column (1 µL of the stock solution) were used. From the obtained total ion chromatogram (TIC) the mass of the compound buspirone $[M+H]^+$= 386.3 was extracted and the data file was uploaded to the Agilent MassHunter qualitative data analysis software with a predefined EIC

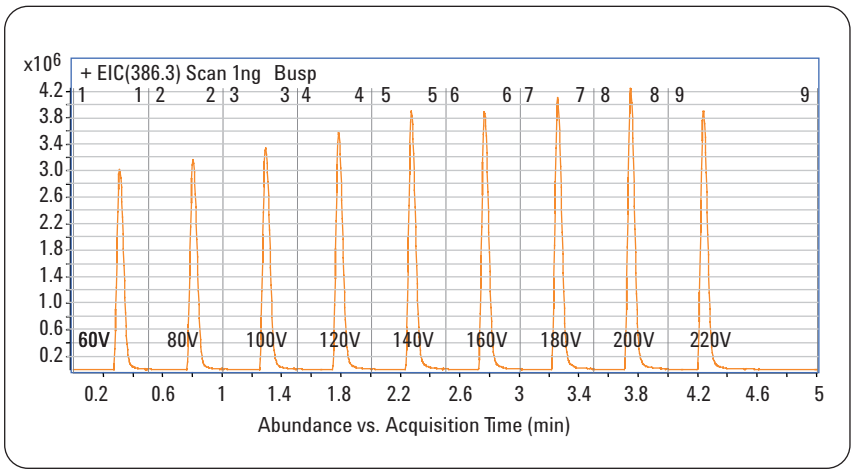

Figure 1
Determination of optimum fragmentor voltage for buspirone $[M+H]^+$= 386.3 by flow injection analysis (FIA) with triple quadrupole MS in scan mode. The figure shows the extracted ion chromatogram of the flow injection analysis.

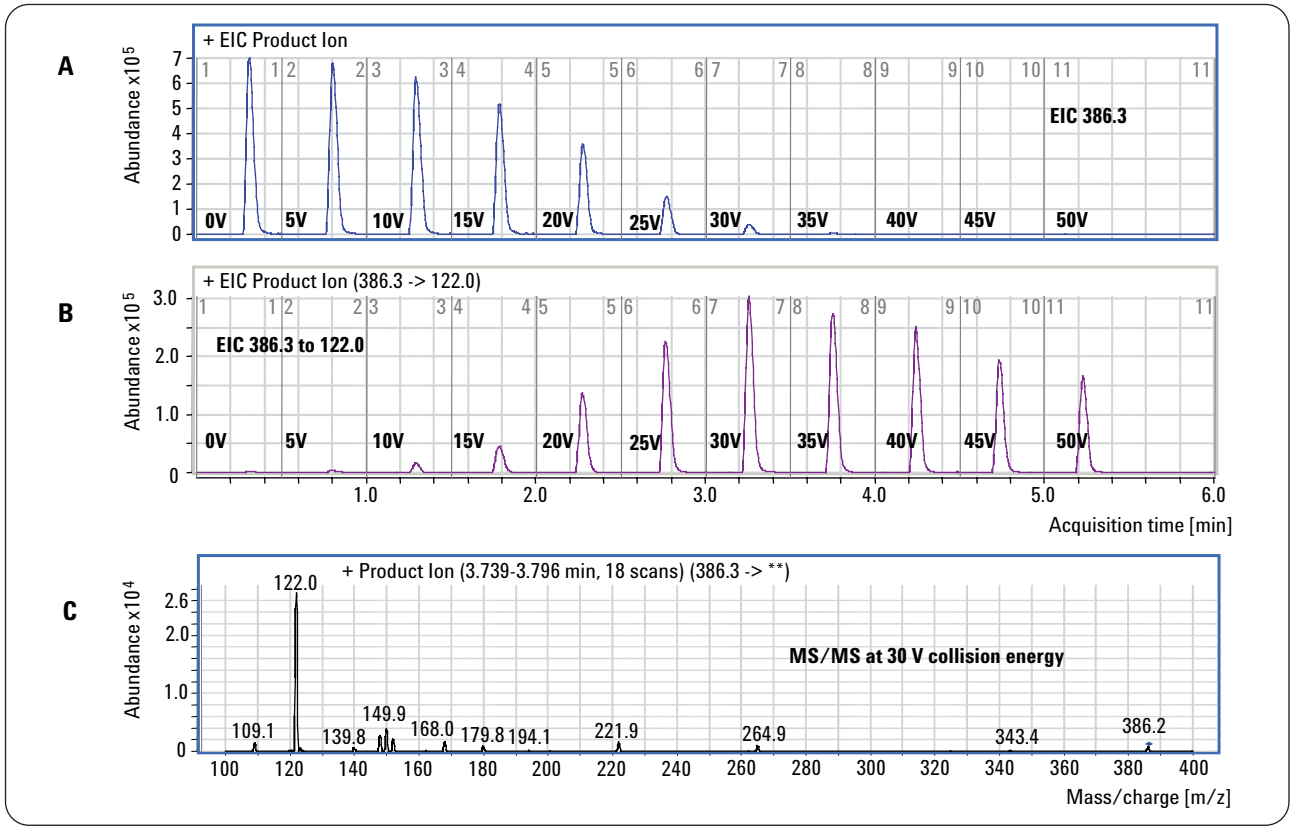

Figure 2
Determination of optimum collision energy for buspirone $[M+H]^+$ = 386.3 by FIA in product ion scan mode.
A) EIC of precursor compound buspirone.
B) EIC of product ion fragmentation of 386.3 to 122.0.
C) MS/MS spectrum of buspirone at optimum conditions.

method (figure 1). Within the outlined time segments the fragmentor voltage was started at 60 V and increased by 20 V up to 220 V. After reviewing the EIC, which was generated in the fast 5 minute flow injection analysis, the optimum fragmentor voltage was determined to be 180 V.

With this optimized fragmentor voltage an additional flow injection experiment was performed to optimize the collision energy. Each time segment of the analysis was operated with increasing collision energy in steps of 5 volts between 0 and 50 V and the used product ion scan mode was operated with the mass of the parent drug buspirone (figure 2). The EIC for the mass of buspirone clearly shows a decreasing signal with increasing collision energy caused by the fragmentation into the product ions (figure 2A). The emerging main fragment has m/z 122.0. The extraction of the transition 386.3 to 122.0 shows an increasing signal for increasing collision energy with an optimum intensity at 30 V (figure 2B). The complete MS/MS spectrum for the fragmentation of the parent ion m/z 386.3 shows the most intense fragment ion at m/z 122.0 (figure 2C). With the obtained optimized MS conditions for fragmentor and collision energy voltages, the MRM transitions

for the parent ion and the chosen product ion was programmed in the method and tested. The signal was obtained at a retention time of 0.95 min within the 2 minute run (figure 3).

To apply the developed method for the detection of the drug molecule, it is important to know the limit of detection (LOD). Therefore, a dilution series was measured down to a concentration of 0.1 pg on column. The limit of detection was measured with signal-to-noise = 3.2 from the run of the sample with the concentration of 100 fg on column (figure 4).

For the measurement of the metabolic stability of buspirone with the optimized LC/MSMS MRM method a series of metabolite samples were prepared by stopping the metabolizing reaction at several time points as described above. After acquisition of the samples the data analysis was performed with the Agilent MassHunter qualitative software with a predefined method for extraction of the defined MRM transition and peak integration.

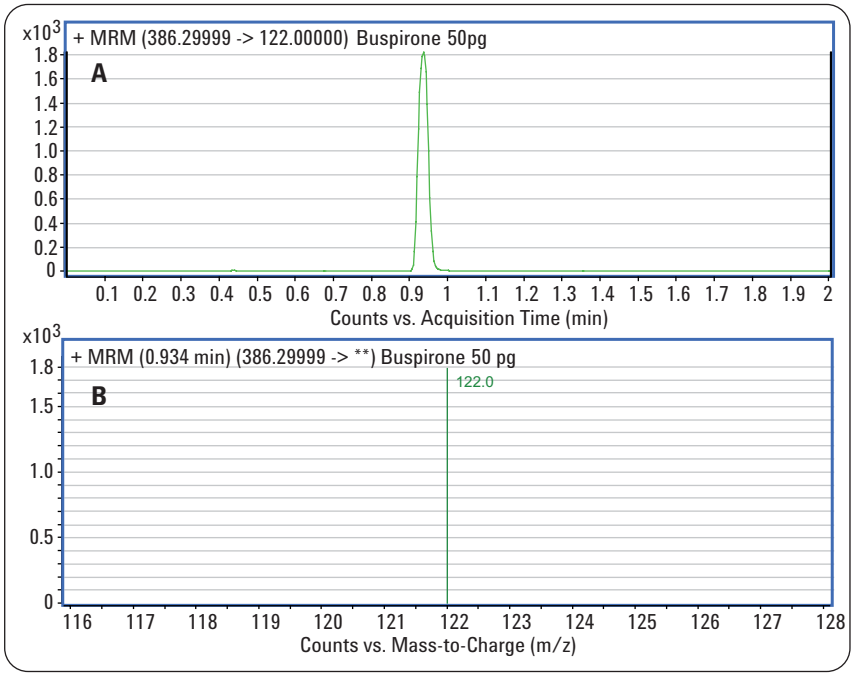

Figure 3
A) MRM transition for 386.3 to 122.0 of Buspirone with 50 pg on column with optimized settings.
B) Product ion spectrum of the MRM experiment.

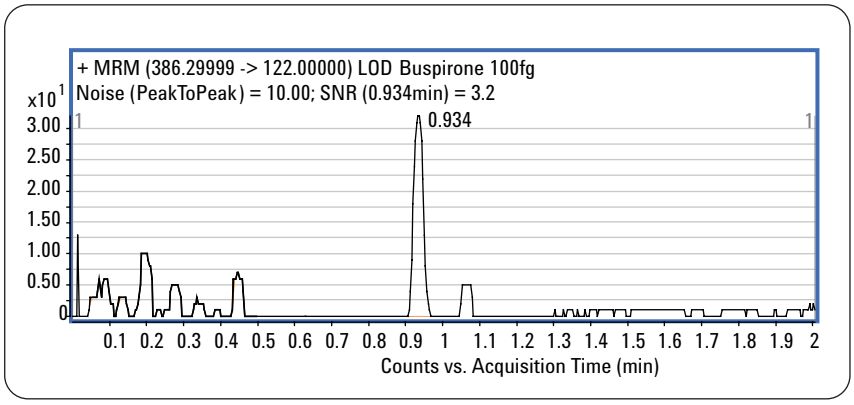

Figure 4
Limit of detection of buspirone with signal to noise (S/N = 3.2) for 100 fg on column by MRM.

The calculated areas show a clear decline during the progress of the metabolizing reaction (table 1). The initial area under the buspirone peak at t=0 was calculated as 100 % and the other areas measured for the various time points were calculated in percent relative to this peak. The metabolic stability curve for buspirone shows a clear decline over the course of the reaction (figure 5). After t=10 minutes of metabolic reaction, 50 % of the parent drug was metabolized. And at the end of the experiment at t=30 minutes, 11 % of the parent drug remained.

Minutes	Area	Area %
0	18013	100
5	12865	72
10	8956	49
15	5615	31
20	4000	22
25	2500	14
30	2039	11

Table 1
Progress of metabolizing reaction of buspirone.

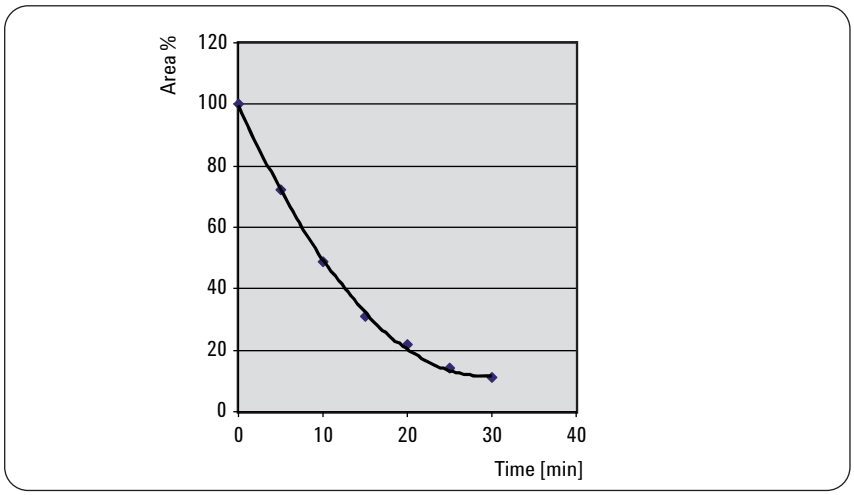

Figure 5
Metabolic stability curve of buspirone.

Conclusion

This Application Note demonstrates the rapid development of an optimized MRM triple quadrupole MS/MS method in combination with a fast, high resolving chromatography for high throughput application. The method was applied for the measurement of the metabolic stability of the pharmaceutical drug buspirone. The relative quantification was calculated from the integrated peak areas and a metabolic stability curve was constructed.

References

1.
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2.
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